Supplementary Methods

Mass Spectrometry and N-terminal Edman Degradation Sequencing

1 µg platelet-purified TSP-1 (Calbiochem/Merck-Millipore, Burlington, MA, USA) was digested with cathepsin G (50 mU/ml) or left untreated and was separated by gradient SDS–PAGE. After silver staining, bands of interest (full-length protein, C-terminal fragment, N-terminal fragment) were excised, digested with trypsin, separated by nanoflow liquid chromatography (1100 Series LC system, Agilent, Palo Alto, CA, USA) followed by MS/MS fragmentation analysis with an ion trap mass spectrometer (XCT-Plus, Agilent).

40 µg TSP-1 were treated with 50 mU/ml cathepsin G and TSP-1 fragments were also separated by 4–20% tris–glycine gel electrophoresis and transferred to a PVDF membrane (Merck KGa). After staining with Ponceau Red, the 160 kDa TSP-1 band was excised and Edman N-terminal degradation sequencing was performed with an ABI 494 Protein Sequencer (service by Tufts University, Boston, MA, USA).

Generation of Purified TSP-1 Isoforms

Platelet-purified TSP-1 (Calbiochem) was treated with cathepsin G (50 mU/ml) and cathepsin G inhibitor (0.2 mM) to obtain 185 kDa and 160 kDa TSP-1. For the full-length protein, cathepsin G and its inhibitor were preincubated for 15 minute at 37°C prior to the addition of purified TSP-1. Then, the sample was incubated for another 45 minute. In contrast, purified TSP-1 was incubated with cathepsin G for 45 minute to generate the 160 kDa isoform. Subsequently, the protease was inhibited for another 15 minutes with cathepsin G inhibitor.

Generation of Recombinant TSP-1 Isoforms

RNA was isolated from ECs, converted into cDNA and the PCR-amplified TSP-1 cDNA fragment was ligated into pcDNA3.1 vector (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). For purification a FLAG tag was added at the C-terminus by site-directed mutagenesis with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA). For the 160 kDa TSP-1 isoform the N-terminus up to amino acid 237 (not counting the signal peptide) was deleted using the site-directed mutagenesis kit. Furthermore, the allelic variant S700 (replacing N700) was generated for the full-length 185 kDa TSP-1 variant with the same mutagenesis cloning kit. TSP-1 expression constructs were then transiently transfected into COS-7 cells via electroporation (1200 µF, 200 V). Thereafter, cells were allowed to grow overnight in Dulbecco's Modified Eagle Medium with 10% FCS. On the next day, cells were washed twice with PBSdef and switched to medium without serum. The cells were allowed to release TSP-1 for another 24 hours before collection of the supernatant for protein purification. After increasing protein concentration by SpeedVac (Thermo Scientific), immunoprecipitations using anti-FLAG M2 Magnetic Beads (Sigma-Aldrich, St. Louise, MO, USA) according to manufacturer's instructions were conducted. TSP-1 contained in 1 ml concentrated culture supernatant was immunoprecipitated with 40 µl of the 50% bead suspension. Elution was performed by competition with 150 ng/µl 3xFLAG peptide (Sigma-Aldrich).

Visualization of NETs by Immunofluorescence Confocal Microscopy

Neutrophils and platelets seeded on glass coverslips were treated with A23187 (4 µM) or A23187 and GSK484 (2 mM) for 3 hours. Cells were fixed with 4% paraformaldehyde, permeabilised (0.2% Tween20 in PBS), blocked (1.5% BSA and 0.1% sodium azide in PBS) and incubated with primary antibodies: rabbit anti-TSP-1 (Abcam, Cambridge, UK) at 1:100 and mouse anti-CD66b (clone 80H3, Immunotech, Montreal, Quebec, Canada) at 1:100 dilution, which were detected with Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 555 donkey anti-mouse IgG secondary antibodies at 1:1000 dilution (Invitrogen/Thermo Fisher Scientific), respectively. Furthermore, platelets were concomitantly stained with mouse anti-CD41-PB (BioLegend, San Diego, CA, USA) at 1:200 dilution. Hoechst 33342 DNA stain (Invitrogen/Thermo Fisher Scientific) was applied at 1:1000 dilution for 10 minutes. Specimens were mounted in Fluoromount G (Southern Biotech, Birmingham, AL, USA) and analyzed with a 63x objective of an LSM700 confocal microscope (Carl Zeiss MicroImaging, Inc., Oberkochen Germany).

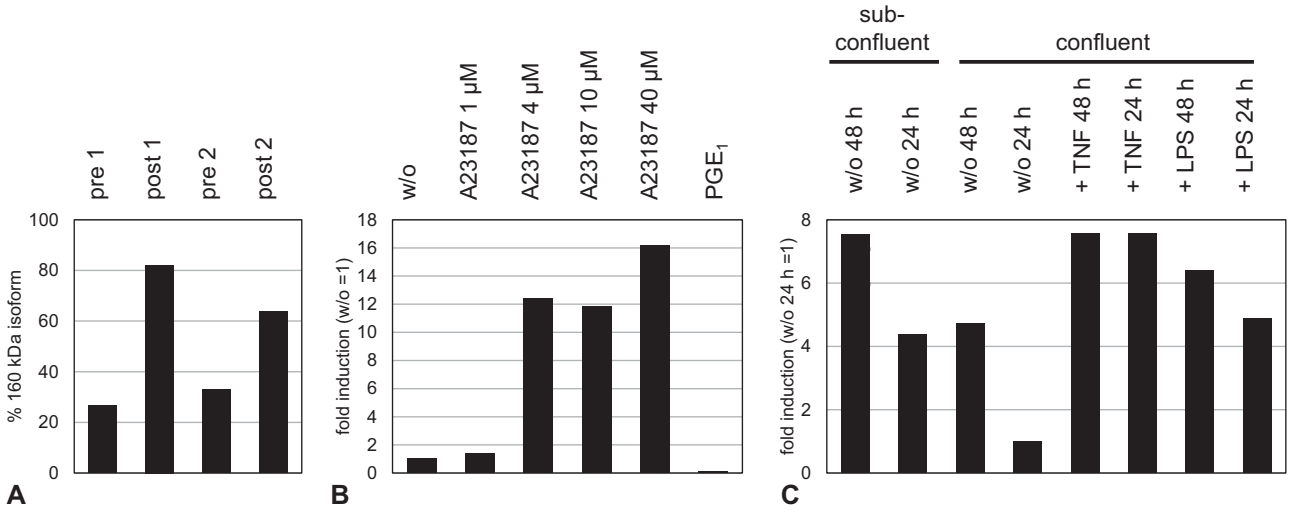
Analysis of TSP-1 Polymerisation by SDS-agarose Discontinuous Gel Electrophoresis

SDS-agarose discontinuous gel electrophoresis was performed essentially as described[1] with some modifications. 1.5 mm gels of medium (1.6%) and low (1.2%) resolution (LGT agarose type VII, Sigma-Aldrich) were prepared in 26 × 15 cm cassettes. A stacking gel consisting of 0.8% Seacam HGT (P) agarose (Lonza Rockland Inc., Rockland, ME, USA) of 1.5 cm length was added. The 160 and 185 kDa TSP-1 isoforms were either recombinantly produced or proteolytically generated from platelet-purified TSP-1. Proteins were then exposed to 5 or 10 mM of EDTA (pH 7.0) at 37°C for 2 hours. After heating for 20 minutes at 56°C samples were loaded and electrophoresis was performed at 65 V for ~1 hour, until the dye had left the slots. Afterwards the empty slots were filled with agarose, the voltage was lowered to 55 V and the electrophoresis was performed overnight until the tracking dye had reached the end of the gel. TSP-1 complexes were transferred to nitrocellulose filters by electroblotting at 1.95 A for 4 hours using transfer buffer (0.05 M phosphate pH7.4, 0.04 M SDS, without methanol). After blocking in ultrahigh heated, low fat commercial milk (with no additions) for 20 minutes the filters were incubated with a 1:500 dilution of anti-human TSP-1 antibody (Ab11, Thermo Fisher Scientific). Following 3 washing steps for 10 minutes in milk the filters were incubated for 1.5 hours with secondary antibody (1:50000 dilution of goat anti-mouse IgG antibody, peroxidase labeled, Invitrogen/Thermo Fisher Scientific). After the

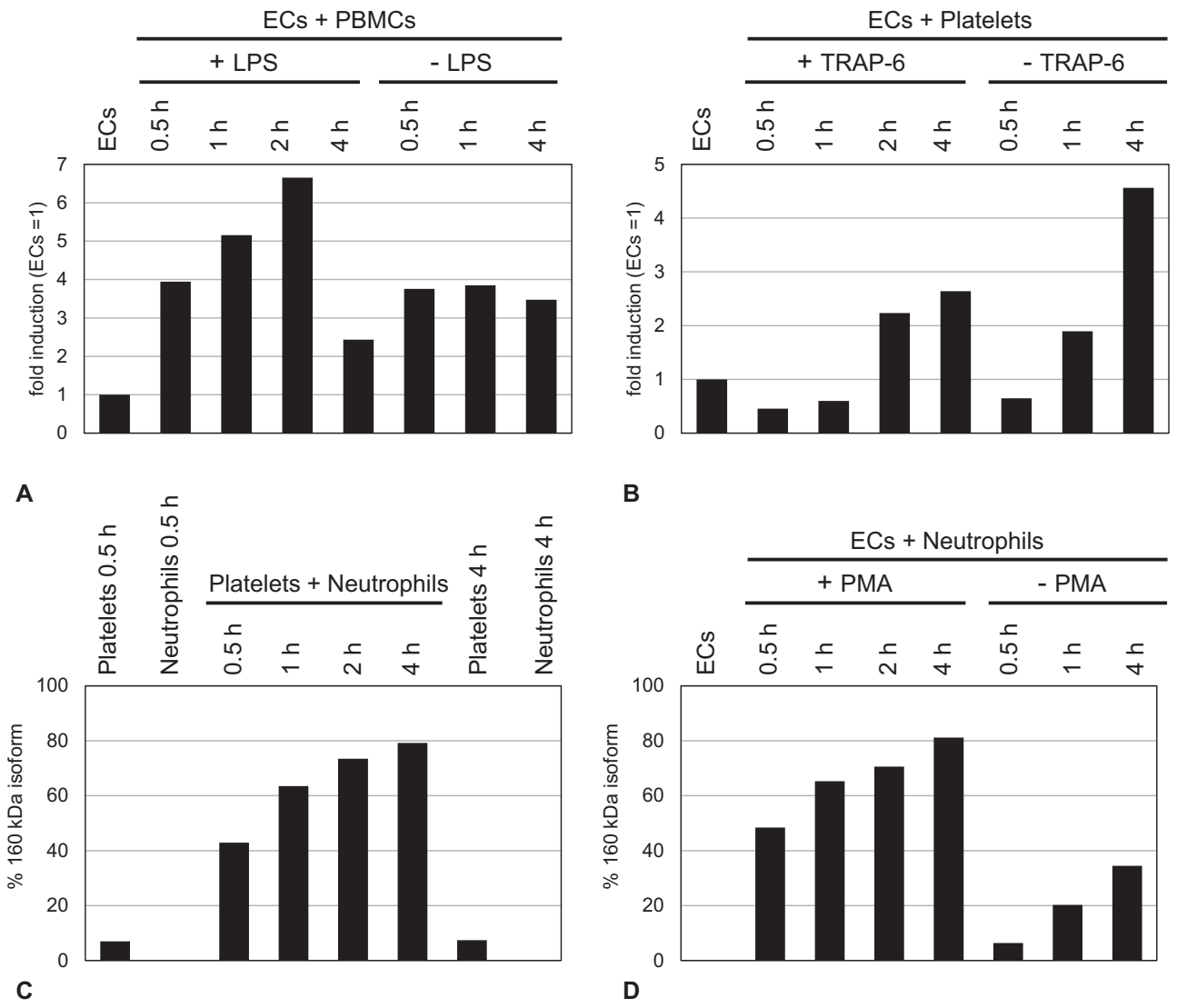
last 3 washing steps in milk the filters were rinsed under running tap water and thereafter placed into the video detection system with cooled CCD camera and Alpha Ease software (Alpha Innotech, San Leandro CA, USA). Filters were overlaid with 3 ml solution of luminol (Lumi-Light Plus, Roche, Darmstadt, Germany) and exposure times around 17 minutes were applied.

Ethical Approval

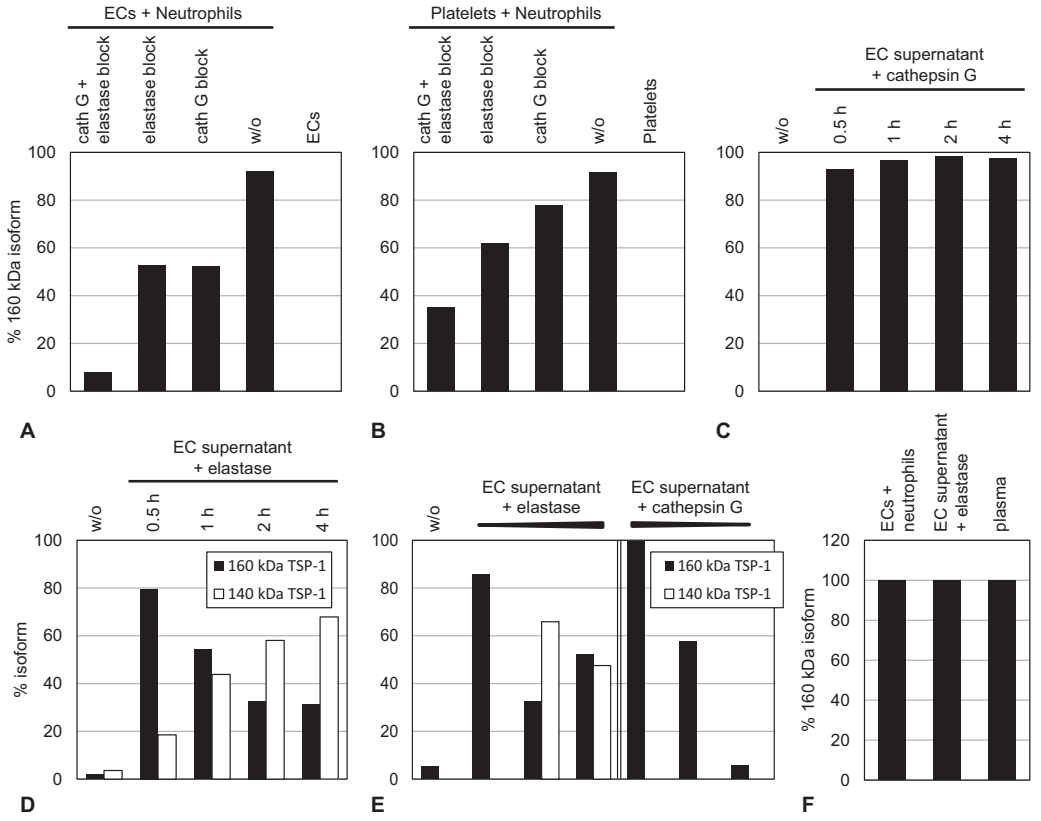
The isolation of human cells was approved by the local ethics committee (no. 1123/2009 and 791/2010). All patients and controls gave written informed consent.
Withdrawal of mouse blood was approved by the ethics committee for animal experimentation (no. 0383-WF/V/3b/2015 and 0270-WF/V/3b/2017).



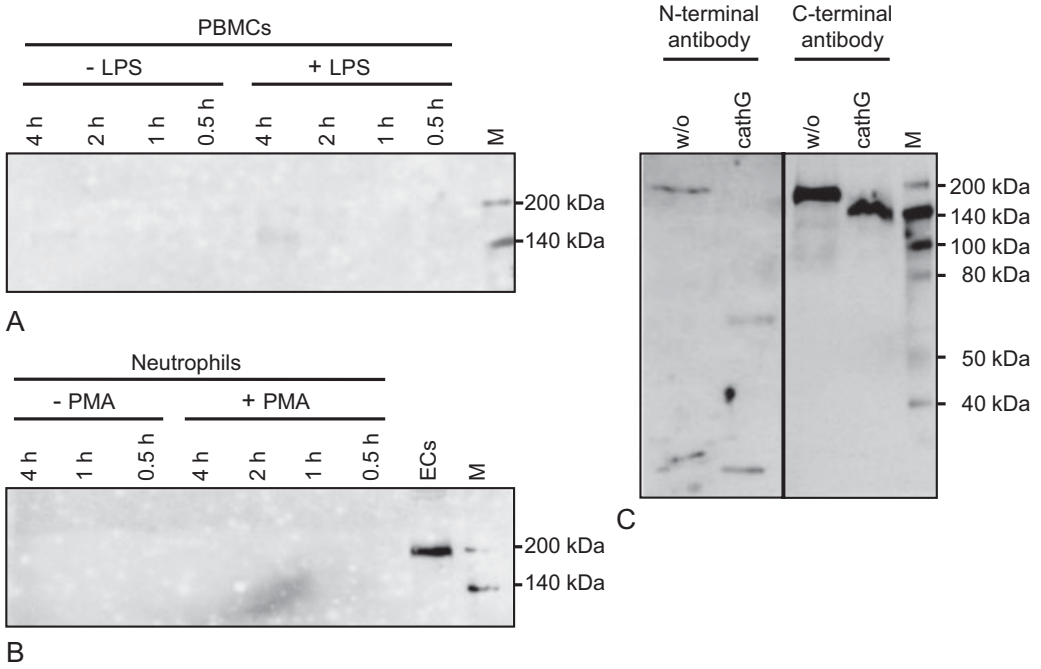
Supplementary Fig. S1 Densitometric quantitation of thrombospondin-1 (TSP-1) in immunoblots of ►**Fig. 1**. TSP-1 protein bands were quantitated using the *Fiji*-version of *ImageJ* software for (**A**) ►**Fig. 1A**, (**B**) ►**Fig. 1B** and (**C**) ►**Fig. 1C**. The fraction of the 160-kDa TSP-1 isoform is given for (**A**), whereas the stimulated release of 185 kDa TSP-1 as compared with untreated control is quantitated in (**B**) and (**C**).



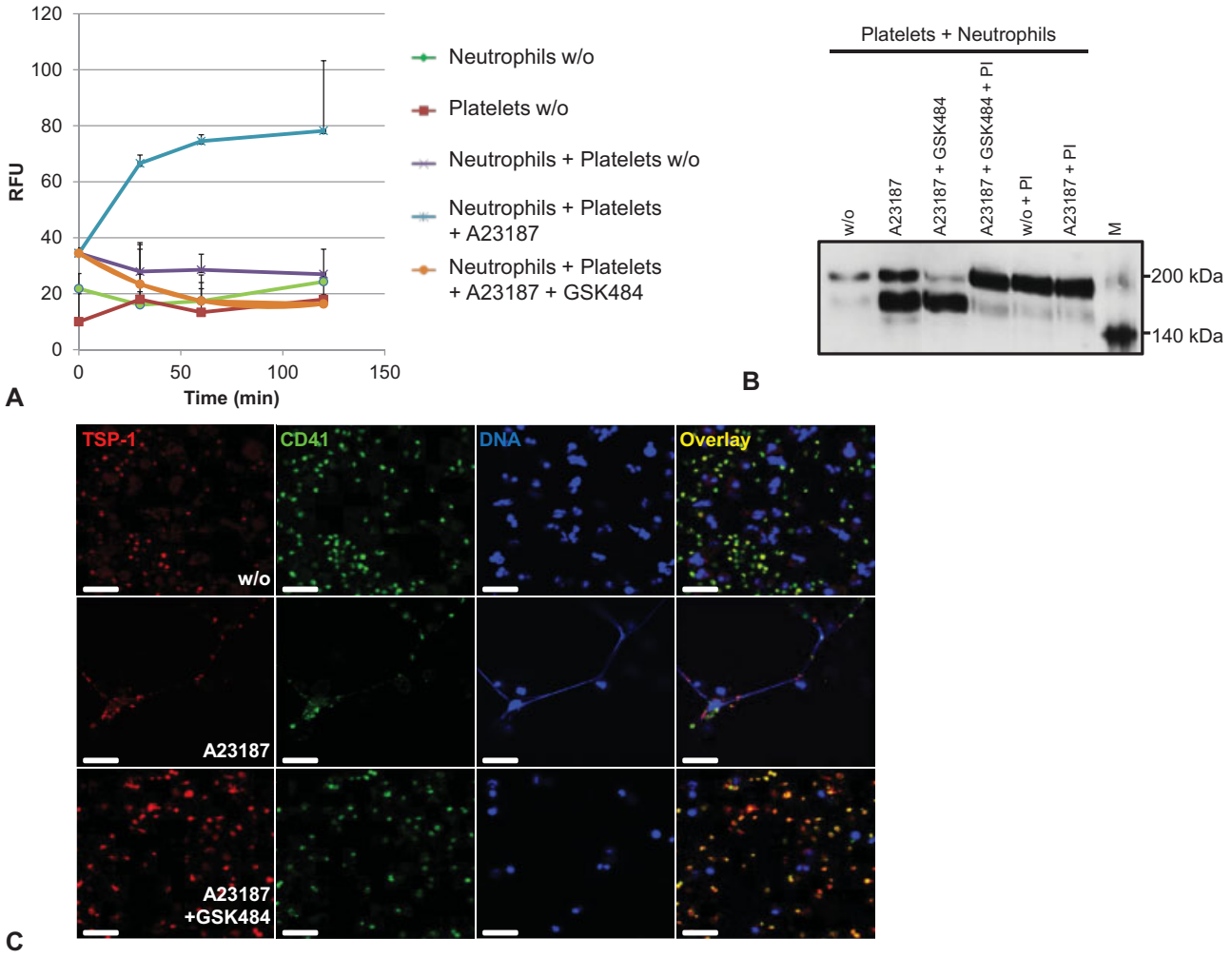
Supplementary Fig. S2 Densitometric quantitation of thrombospondin-1 (TSP-1) in immunoblots of ►**Fig. 2**. TSP-1 protein bands were quantitated using the *Fiji*-version of *ImageJ* software for (**A**) ►**Fig. 2A**, (**B**) ►**Fig. 2B**, (**C**) ►**Fig. 2C** and (**D**) ►**Fig. 2D**. Changes in the expression level of 185 kDa TSP-1 as compared with untreated control are illustrated in (**A**) and (**B**); the percentage of 160 kDa TSP-1 is given for (**C**) and (**D**).



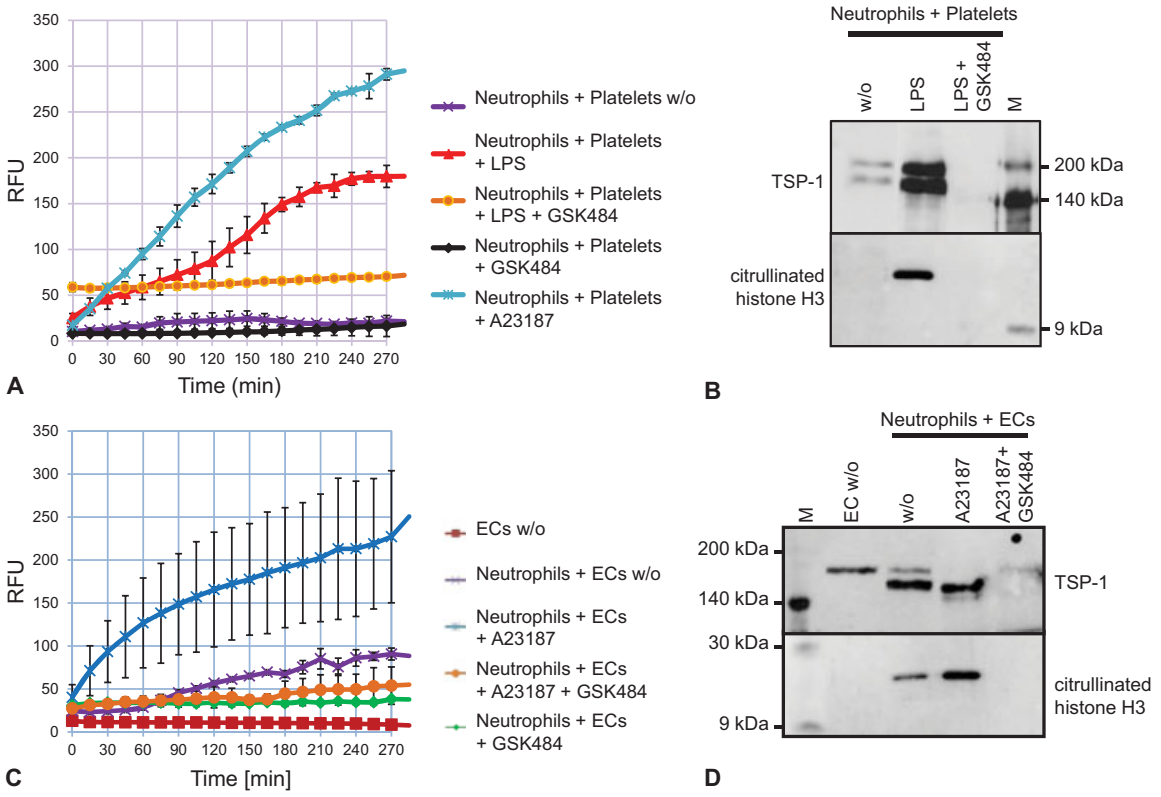
Supplementary Fig. S3 Densitometric quantitation of thrombospondin-1 (TSP-1) in immunoblots of ►**Fig. 3**. TSP-1 protein bands were quantitated using the *Fiji*-version of *ImageJ* software for (**A**) ►**Fig. 3A**, (**B**) ►**Fig. 3B**, (**C**) ►**Fig. 3C**, (**D**) ►**Fig. 3D**, (**E**) ►**Fig. 3E** and (**F**) ►**Fig. 3F**. TSP-1 conversion from 185 to 160 kDa is given for (**A, B, C and F**), whereas (**D**) and (**E**) illustrate the percentage of the 160 as well as the 140 kDa isoform.



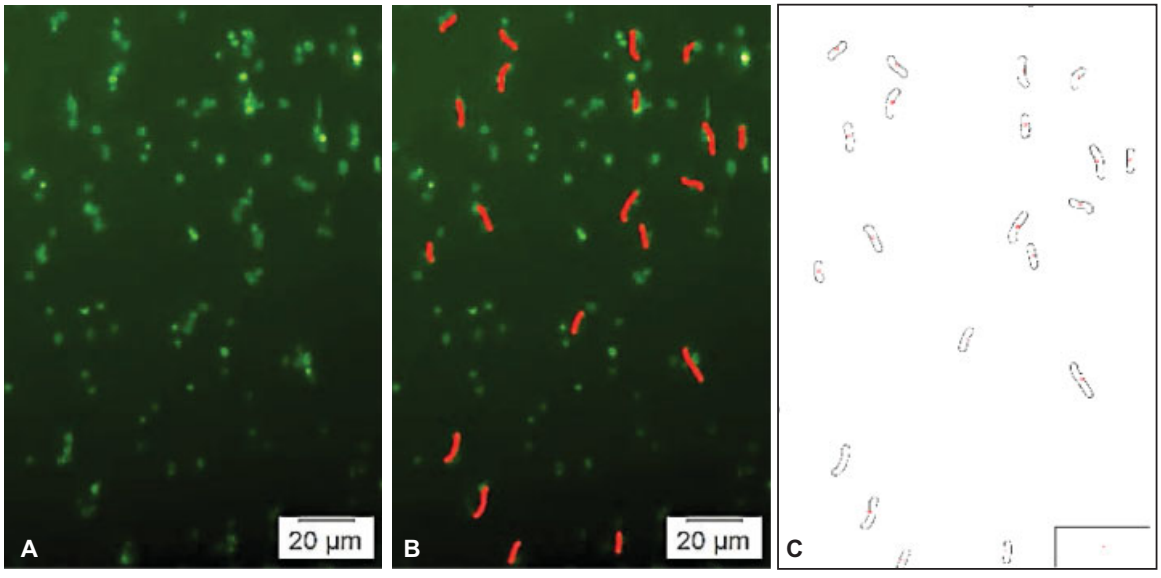
Supplementary Fig. S4 Characterization of thrombospondin-1 (TSP-1) isoform generation. In addition to co-cultures, TSP-1 secretion in separate peripheral blood mononuclear cell (PBMC) and neutrophil populations was assessed for control. Freshly isolated human (**A**) PBMCs or (**B**) neutrophils were cultured for 0.5, 1, 2 and 4 hours before collection of the supernatant and detection of TSP-1 via immunoblotting with Ab11. Where indicated (+) lipopolysaccharide (LPS) was added at 1 µg/mL or phorbol myristate acetate (PMA) at 100 ng/mL to activate PBMCs or neutrophils, respectively. An endothelial culture supernatant (endothelial cells [ECs]) was loaded as positive control. (**C**) Detection of N- and C-terminal protein domains after TSP-1 fragmentation by cathepsin G was performed with domain-specific antibodies. Serum-free EC supernatant was left untreated (w/o) or was exposed to cathepsin G (10 mU/mL) for 30 minutes at 37°C prior to immunostaining with two distinct antibodies, either specific for the N-terminal or the C-terminal domain of TSP-1. M, biotinylated protein marker.



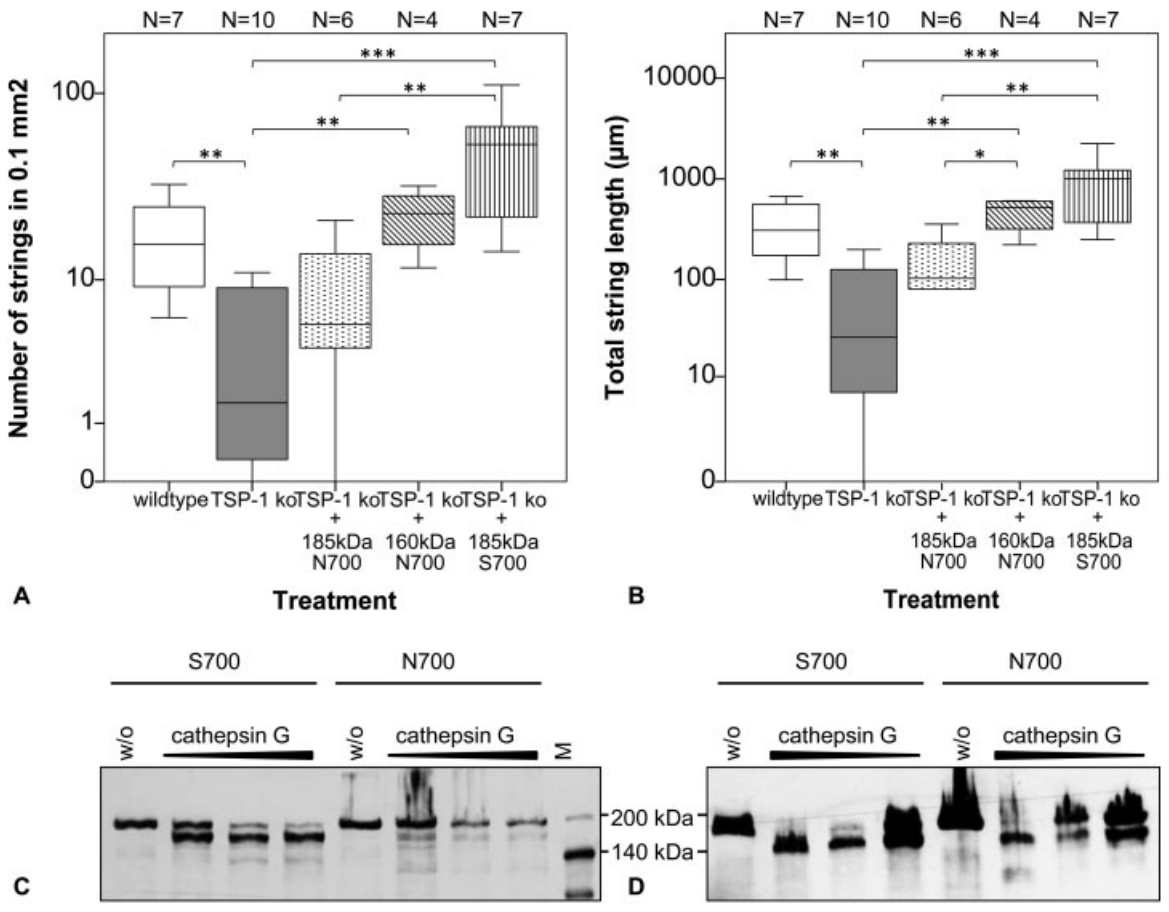
Supplementary Fig. S5 Neutrophil-mediated proteolysis of thrombospondin-1 (TSP-1) in the presence of neutrophil extracellular trap (NET) inhibitor. (**A**) Co-cultures of 1×10^7 platelets with 1×10^5 neutrophils were left untreated, stimulated with 4 µM A23187 or challenged with 4 µM A23187 in the presence of 2 mM GSK484 to block NETosis. A, After 0, 30, 60 and 120 minutes of incubation, NETs were dismantled by treatment with 500 mU/mL micrococcal nuclease for 10 minutes before supernatant was retrieved and citrullinated histone H3 was determined by enzyme-linked immunosorbent assay (ELISA). (**B**) Co-cultures of platelets and neutrophils were supplemented with 50 µM cathepsin G inhibitor and 1 mM elastase inhibitor (protease inhibitors [PI] were given concomitantly with GSK484), then pre-incubated for 30 minutes before stimulation with 4 µM A23187 for 60 minutes. TSP-1 was detected in the nuclease-treated culture supernatant by immunoblotting with Ab11. (**C**) Co-cultures seeded and stimulated on cover slips for 3 hours were fixed, permeabilized and immunostained for TSP-1 (red), the platelet marker CD41 (green) and deoxyribonucleic acid (DNA) (blue). Scale bar: 20 µm.



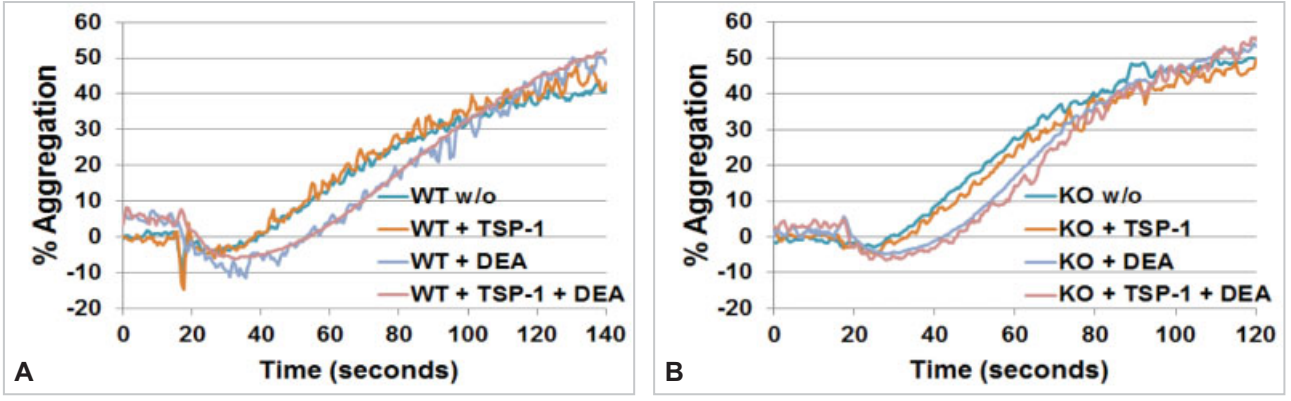
Supplementary Fig. S6 Neutrophil-mediated proteolysis of thrombospondin-1 (TSP-1) in distinct settings of neutrophil extracellular trap (NET) induction. (**A**, **B**) Co-cultures of neutrophils and platelets were left untreated or stimulated with 300 ng/mL lipopolysaccharide (LPS) in the absence or presence of the NETosis inhibitor GSK484 at 2 mM. Stimulation with 4 µM A23187 is shown for comparison. (**C**, **D**) Co-cultures of neutrophils and endothelial cells were comparably left untreated, stimulated with 4 µM A23187 or challenged with 4 µM A23187 in the presence of 2 mM GSK484 to block NET formation. (**A** and **C**) Release of neutrophil deoxyribonucleic acid (DNA) was assessed by incorporation of Sytox Green dye and measurement of relative fluorescence units (RFUs) over 5 hours. Mean and standard deviation of two experiments are shown. (**B** and **D**) Occurrence of TSP-1 isoforms (upper panel) and citrullination of histone H3 (lower panel) was determined at 120 minutes by immunoblotting.



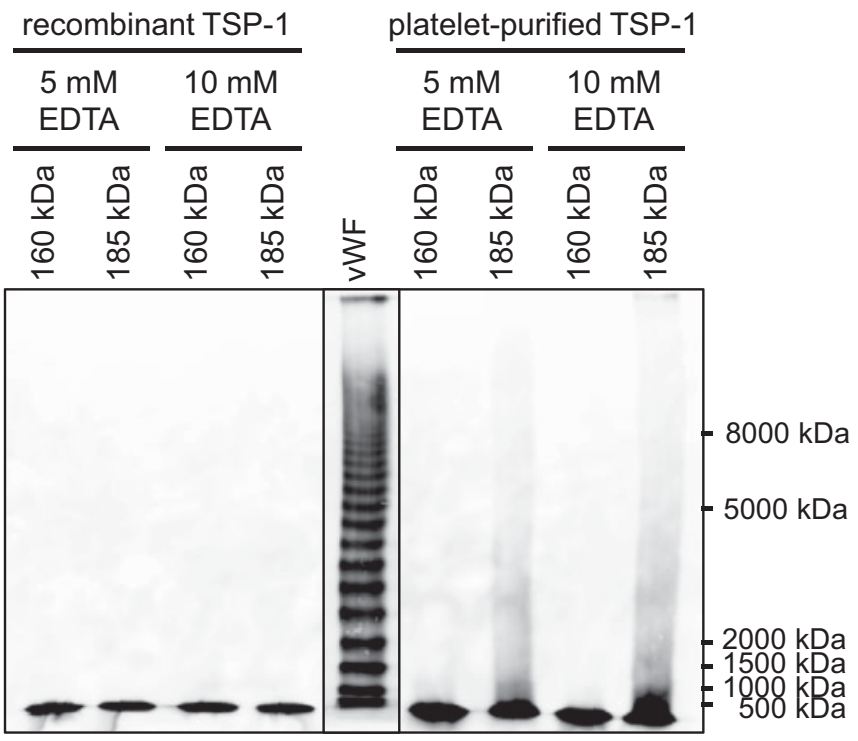
Supplementary Fig. S7 Illustration of automated analysis of platelet strings. Mouse blood with fluorescently labelled platelets was perfused over collagen-coated slides in an *ibidi* flow chamber for 7 minutes at 7 dyne/cm^2. Platelets were visualized with an Olympus IX83 microscope and (**A**) 10 images per sample were taken within 2 minutes with an Orca Flash 4.0 camera using the *CellSens Dimensions* software. The number of adherent platelet strings and the mean length of formed platelet strings were analysed with the *Fiji*-version of *ImageJ* software. (**B**) Platelet strings were first manually labelled (red lines) requiring a minimum of three connected platelets per string. (**C**) Composite colour images were then split into red, green and blue channels and the red channel was subjected to automated thresholding using the *MaxEntropy* algorithm followed by the 'analyse particles' routine using a minimum size of 50 pixels. The number and length of all identified strings were recorded. Scale bar: 20 µm.



Supplementary Fig. S8 Comparison of recombinant thrombospondin-1 (TSP-1) isoforms. (**A** and **B**) Blood retrieved from wild-type or TSP-1 knockout (ko) mice was supplied with anti-GPIbβ antibody to fluorescently label platelets and was perfused over collagen-coated slides in an *ibidi* flow chamber for 7 minutes at 7 dyne/cm^2. TSP-1 ko blood was either left untreated or was substituted with 1 µg/mL of recombinantly produced, purified 160 kDa and 185 kDa TSP-1 protein with N700 or S700 configuration. The number of adherent platelet strings (**A**) and the total length of formed platelet strings (**B**) were analysed with the *Fiji*-version of *ImageJ* software. Boxplots illustrate the data distribution of 4 to 10 independent experiments (representing blood from individual mice); statistically significant differences between groups were assessed by Mann–Whitney U test (SPSS 23.0). (**C** and **D**) The purified 185 kDa molecules of TSP-1 N700 and S700 were subjected to increasing doses of cathepsin G (2.5–10 mU/mL for 40 minutes at 37°C) in two separate experiments, prior to gel electrophoresis and immunoblotting with Ab11. M, biotinylated protein marker.



Supplementary Fig. S9 Comparison of wild-type (WT) and thrombospondin-1 (TSP-1) knockout (KO) platelets in light transmission aggregometry. Blood was drawn from the vena cava of (**A**) WT and (**B**) TSP-1 KO mice, stabilized with acid-citrate-dextrose anticoagulant, and platelets were isolated in Tyrode's Hepes buffer. Aggregation of platelets was measured by light transmission aggregometry using a PAP8 Profiler (moeLab GmbH, Langenfeld, Germany). Where indicated, platelets were pre-incubated for 15 minutes with purified, full-length 185 kDa TSP-1 (5 µg/mL) and/or with the nitric oxide donor diethylamine (DEA) NONOate (10 µM DEA) for 30 seconds. Thereafter, the platelet agonist thrombin (0.2 U/mL) was immediately added and aggregation was recorded. Shown are the results from one of three comparable experiments.



Supplementary Fig. S10 Polymerization properties of thrombospondin-1 (TSP-1) isoforms. The 160 and 185 kDa TSP-1 isoforms were either recombinantly produced or proteolytically generated from platelet-purified thrombospondin-1. Proteins were then exposed to 5 or 10 mM of ethylenediaminetetraacetic acid (EDTA) (pH 7.0) at 37°C for 2 hours before detection of multimers by sodium dodecyl sulphate (SDS)-agarose gel electrophoresis. For size assessment of TSP-1 polymers, von Willebrand factor (vWF) multimers were comparably loaded. TSP-1 and vWF were visualized on Western blots by consecutive high-sensitivity chemiluminescence detection with specific antibodies.

References

1 Budde U, Schneppenheim R, Eikenboom J, et al. Detailed von Willebrand factor multimer analysis in patients with von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). J Thromb Haemost 2008;6(05):762–771